

## The *Lactobacillus plantarum* Eno A1 Enolase Is Involved in Immunostimulation of Caco-2 Cells and in Biofilm Development

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### Abstract

The role of probiotics in prevention and treatment of a variety of diseases is now well assessed. The presence of adhesive molecules on the cell surface of probiotics has been related to the ability to confer health benefit to the host. We have previously shown that the enolase EnoA1 of *Lactobacillus plantarum*, one of the most predominant species in the gut microbiota of healthy individuals, is cell surface-expressed and is involved in binding with human fibronectin and plasminogen. By means of comparative analysis between *L. plantarum* LM3 (wild type) and its isogenic LM3-CC1 ( $\Delta$ enoA1) mutant strain, here we show that EnoA1 affects the ability of this bacterium to modulate immune response as determined by analysis of expression of immune system molecules in Caco-2 cells. Indeed, we observed induction of TLR2 expression in cells exposed to *L. plantarum* LM3, while no induction was detectable in cells exposed to LM3-CC1. This difference was much less consistent when expression of TLR4 was determined in cells exposed to the two strains. Pro-inflammatory (IL-6) and anti-inflammatory cytokines (IL-10, TGF- $\beta$ ), and the antimicrobial peptide HBD-2 were induced in Caco-2 cells exposed to *L. plantarum* LM3, while lower levels of induction were detected in cells exposed to LM3-CC1. We also analyzed the ability to develop biofilm of the two strains, and observed a decrease of about 65 % in the development of mature biofilm in LM3-CC1 compared to the wild type.

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**Keywords***L. plantarum* LM3 • Adhesins • Immunomodulation • Biofilm**1 Introduction**

The intestinal epithelium, once considered simply a physical barrier, is now considered a regulator of intestinal immune homeostasis. In this regard, the interaction of epithelial cells with the microbiota has the key role of mediator of the cross-talk between the epithelium and other cell types in the mucosa (Darab et al. 2011). Indeed, intestinal epithelium cells (IECs) express a wide variety of pattern recognition receptors (PRR) to detect the presence of microbes. The well-characterized PRR expressed by epithelial cells include principally members of the Toll-like (TLR) family, which can trigger expression of a surprising diversity of chemokines, cytokines and effectors of innate and inflammatory response (Sieling and Modlin 2002; Abreu et al. 2003). Immunohistochemical techniques have shown that TLR2 and TLR4 are expressed at low levels by IEC in the human colon tissues of healthy individuals; TLR2 plays a major role in the detection of Gram-positive bacteria via recognition of cell wall PAMPs, including lipoteichoic acid, lipoproteins, and peptidoglycan (Abreu et al. 2005; Kelly and Conway 2005), while TLR4 plays a role in the intestinal mucosal host defense against Gram-negative bacteria and is essential for LPS detection (Vinderola et al. 2005). The intestinal microbiota is largely mutualistic in nature and important for human health. Apart from its well-established role in nutrition, it is important in development of the immune system and maintenance of homeostasis of tolerance and immunity (Himanshu et al. 2001). Administration of indigenous, non-pathogenic probiotic bacteria is a promising strategy to improve immune homeostasis and to maintain host health by normalizing existing undesired immune responses, as in the

case of allergy or autoimmune disease (Vinderola et al. 2005; Adams 2010). Epithelial cells from the small and large intestines make a major distinction between probiotic and pathogens. This distinction comprises the type of cytokines released and the magnitude of the response (Peyrin-Biroulet et al. 2006).

The interest in the innate immune response in inflammatory bowel diseases has led to evaluate the role of defensins, part of a family of antimicrobial peptides with direct bactericidal activity against a wide variety of bacteria (Peyrin-Biroulet et al. 2006; Marian et al. 2009). Human  $\beta$ -defensins (HBDs) are small cationic antimicrobial peptides synthesized by the epithelium to counteract bacterial adherence and invasion. HBDs are expressed in various tissues throughout the body; the best characterized gut defensin is the HBD-2, an inducible peptide, synthesized and secreted by the epithelium, with a strong antimicrobial activity (Vora et al. 2004). Unlike HBD-1, which is produced constitutively, HBD-2 is expressed after adequate stimulation by cytokines and/or bacterial components in epithelial tissues and mononuclear phagocytes. Some probiotic lactobacilli have been shown to induce HBD-2, but they are not affected by the antimicrobial effect of HBD-2, which appear to be specific for pathogens (Schlee et al. 2007). Moreover, a further effect of probiotics on the mucosal barrier function is the ability to induce production of some anti-inflammatory cytokines (e.g. IL-10, TGF- $\beta$ ) and transient production of pro-inflammatory cytokines (e.g. IL-6) (Boirivant and Strober 2007; Li et al. 2006).

The effect of probiotics on modulation of immune response is strain dependent, mainly based on different molecules expressed on cell surface, needed for the cross-talk with epithelial

cells. Among these molecules, adhesins have a critical role in colonization of the intestine, in protection against pathogens through competitive exclusion, and in development of biofilms, important for persistence of probiotic strains in the colon (Macfarlane 2008). Lactic acid bacteria (LAB) of the genus *Lactobacillus* and *Bifidobacterium*, traditionally present in dairy products, have been used for treatment and prevention of gut diseases, and the beneficial effects of some probiotic LAB strains have been assessed by clinical trials (Gareau et al. 2010; Ahrne and Hagslatt 2011). Among these, some strains of *Lactobacillus plantarum*, one of the most predominant species in the human microbiota of healthy individuals, were demonstrated to be good performing probiotic microorganisms (van Baarlen et al. 2009; Kaushik et al. 2009; Lonnermark et al. 2010). We have previously shown adhesion of *L. plantarum* LM3 to Caco-2 cells, and identified enolase as a surface-expressed protein involved in adhesion to fibronectin and to plasminogen (Castaldo et al. 2009; Vastano et al. 2013). Enolases are highly conserved proteins, with an essential role in central metabolism and with moonlighting functions in many microorganisms and in different eucariotic cell types. Surface-expressed enolases are involved in pathogenesis of many microorganisms, either in the role of adhesion molecules for different host receptors, or as molecules involved in the host immuneresponse (Veiga-Malta et al. 2004; Adrian et al. 2015).

The aim of this study was to evaluate the potential immunomodulatory effects of *L. plantarum* LM3 and its adhesion-defective mutant LM3-CC1, carrying a deletion in the *enoA1* gene, coding the enolase EnoA1. The analysis was performed by determining the expression level of some immunomodulatory molecules on Caco-2 cells exposed to *L. plantarum* wild type and mutant strain. Moreover, the role of the surface expressed enolase in the *L. plantarum* ability to develop biofilm, the sessile form adopted by microorganisms to persist in the colon, was also evaluated.

## 2 Methods

### 2.1 Cell Lines, Media and Bacterial Strains

The human colon adenocarcinoma Caco-2 cells, (from the American Type Culture Collection ATCC), were maintained in Dulbecco's Modified Eagle's Medium (DMEM). Growth media were supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS), 1 % (v/v) non-essential amino acids, and a standard mixture of antibiotics (100 U ml<sup>-1</sup> penicillin, 100 µg ml<sup>-1</sup> streptomycin) and 2 mmol l-glutamine at 37 °C under an atmosphere of 5 % CO<sub>2</sub>. All reagents were purchased from Gibco-BRL (UK). Caco-2 cells differentiated into enterocytes after 15–20 days in culture. The cells were grown as monolayers in 75 cm<sup>2</sup> flasks (Greiner, Frickenhausen, Germany) at 37 °C in a 5 % CO<sub>2</sub>-95 % air atmosphere with 90 % humidity. *L. plantarum* LM3 (Muscariello et al. 2013) and LM3-CC1 (Veiga-Malta et al. 2004) strains were cultured at 30 °C in MRS broth supplemented with either 0.2 % or 2 % glucose. When needed, erythromycin (5 µg ml<sup>-1</sup>) was added to *L. plantarum* LM3-CC1 cultures.

### 2.2 Stimulation of Caco-2 Cells

Caco-2 cells were cultured in six-well plates (Becton Dickinson GmbH, Heidelberg, Germany) at a concentration of 10<sup>6</sup> cells/ml for 12 h. Caco-2 cells were incubated with *L. plantarum* LM3 or LM3-CC1 (100 bacteria/cell) for 3, 6, and 24 h in 5 % CO<sub>2</sub> at 37 °C. Infected monolayers were centrifuged at 6000×g for 5 min to favor adhesion. After the specific time of treatment, infected monolayers were washed three times with DMEM to remove non-adherent bacteria. Untreated Caco-2 cells served as controls. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (ATCC, Manassas, USA),

according to the manufacturer's protocol. The culture media were then collected, cells were washed twice with phosphate-buffered saline (PBS) and harvested with High Pure RNA Isolation Kit (Roche Diagnostics, Milano, Italy) from Caco-2 cells infected or not with *L. plantarum*.

### 2.3 Real-Time PCR

To study expression of TLR2, TLR4, HBDs, IL-6, TGF- $\beta$  and IL-10 in Caco-2 cells, Real-Time PCR was performed on RNA extracted from the cells challenged with *L. plantarum* LM3 and LM3-2. Real-time PCR analyses were performed in a fluorescence temperature cycler (LC Fast Start DNA Master SYBR Green, Roche Diagnostics) (LightCycler 2.0 Instrument, GmbH, Mannheim, Germany) according to the manufacturer's instructions. cDNA corresponding to 10 ng of RNA served as a template in a 20  $\mu$ l reaction mixture containing 3 mM MgCl<sub>2</sub>, 0.5  $\mu$ M of each primer and 1X LightCycler-FastStart DNA Master SYBR Green I mix. Sequences of the oligonucleotides used for amplification and related programs are shown in

Table 1. At the end of each run, melting curve profiles were achieved by cooling the sample to 65 °C for 15 s and then heating slowly at 0.20 °C/s up to 95 °C, with continuous measurement of fluorescence to confirm amplification of specific transcripts. Cycle-to-cycle fluorescence emission readings were monitored and analyzed using LightCycler software (Roche Diagnostics GmbH). Melting curves were generated after each run to confirm amplification of specific transcripts. As an internal control gene we used the  $\beta$ -actin coding gene, one of the most commonly used housekeeping genes. All reactions were carried out in triplicate, and the relative expression of a specific mRNA was determined by calculating the fold change relative to the  $\beta$ -actin control. The fold change of the test gene mRNA was obtained with a Biorad software, using the efficiency of each primer as calculated by the dilution curve.  $\Delta$ Ct = the difference of Ct between stimulated and control samples (Valaes and Repa 2005).

The specificity of the amplification products was verified by subjecting the amplification products to electrophoresis on a 2 % agarose gel and visualization by ethidium bromide staining.

**Table 1** Primers used in the study

Gene	Sequence sense and antisense	Conditions	bp
HBD-1	5'-TTG TCT GAG ATG GCC TCA GGT GGT AAC-3'	15'' at 95 °C, 5'' at 60 °C,	200
	5'-TTT CAC TTC TGC GTC ATT TCT TCT GG-3'	10'' at 72 °C for 45 cycles	
HBD-2	5'-GGATCCATGGGTATAGGCGATCCTGTTA-3'	5'' at 94 °C, 6'' at 63 °C,	198
	5'-AAGCTTCTCTGATGAGGGAGCCCTTTCT-3'	10'' at 72 °C for 50 cycles	
HBD-3	5'-AGC CTA GCA GCT ATG AGG ATC-3'	15'' at 95 °C, 5'' at 60 °C,	206
	5'-CTT CGG CAG CAT TTT GCG CCA-3'	10'' at 72 °C for 45 cycles	
TLR-2	5'-TCGGAGTTCTCCAGTTCTCT-3'	30'' at 95 °C, 30'' at 59 °C,	175
	5'-TCCAGTGCTTCAACCCACAA-3'	30'' at 72 °C for 50 cycles	
TLR-4	5'-CGTGGAGACTTGGCCCTAAA-3'	30'' at 95 °C, 30'' at 59 °C,	301
	5'-TTCACACCTGGATAAATCCAGC-3'	30'' at 72 °C for 50 cycles	
IL-6	5'-ATGAACTCCTTCTCCACAAGCGC-3'	5'' at 95 °C, 13'' at 56 °C,	628
	5'-GAAGAGCCCTCAGGCTGGACTG-3'	25'' at 72 °C for 40 cycles	
IL-10	5'-CTTTAAGGGTTACCTGGGTTGCCAAG-3'	5'' at 95 °C, 9'' at 60 °C, 18''	223
	5'-ATTAAGGCATTCTTCACTGCTCCAC-3'	at 72 °C for 35 cycles	
TGF- $\beta$ <sub>1</sub>	5'-CCGACTACTACGCCAAGGAGGTCAC-3'	5'' at 95 °C, 8'' at 60 °C, 16''	439
	5'-AGGCCGGTTCATGCCATGAATGGTG-3'	at 72 °C for 35 cycles	
$\beta$ -actin	5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3'	33 cycles 94 °C for 30''	661
	5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3'	63 °C for 30'', 72 °C for 30''	

## 2.4 Microtiter Plate Assay of *L. plantarum* Biofilm Formation (for Biofilm Quantization)

The microtiter plate assay measures the level of cells adhering to the surface of microtiter plate wells. The assay was performed as described by Hamon and Lazizzera (2001), with the following modifications. Cells were grown at 30 °C in MRS medium supplemented with 2 % or 0.2 % (wt/vol) glucose under static conditions. Overnight cultures of *L. plantarum* LM3 (wt) and LM3-CC1 were diluted in fresh medium to obtain optical density (OD<sub>600</sub>) of 0.05. Two hundred ml of each diluted cell suspension were inoculated into the wells of a 96-well (flat-bottom) cell culture plate (Falcon 35-3072). Wells containing un-inoculated growth medium were used as negative controls. Plates were incubated at 30 °C for 24 h in static condition. Media and unattached cells were removed by rinsing with 200 ml of sterile wash buffer (150 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 100 mM potassium-phosphate buffer pH 7.0, 34 mM Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>, 1 mM MgSO<sub>4</sub>). Adherent bacteria were stained with 200 µl of 1 % (wt/vol) crystal violet for 15 min at room temperature. After two rinses with 200 µl of sterile distilled water each time, the bound dye was extracted from the stained cells by using 200 µl of 80 % ethanol, 20 % acetone. Biofilm formation was quantified by measuring the OD<sub>570</sub> for each well using a Bio-Rad model 680 microplate reader.

## 2.5 Statistical Analysis

The significance of the differences in the results of each test compared to the relative control values was determined with the Student t-test. Values of  $P < 0.05$  were considered statistically significant. The data are presented as means  $\pm$  standard deviation (SD) of three independent experiments.

## 3 Results

### 3.1 Expression of TLR-2 and TLR-4 in Caco-2 Cells Upon Exposure to *L. plantarum* LM3 and LM3-CC1

To study the immunomodulatory effect of *L. plantarum* on Caco-2 cells we first determined the expression of the TLR-2 and TLR-4 mediators. The LM3 wild type induced of about 3 fold the expression of TLR-2 in Caco-2 cells after 3 h of exposure; the effect decreased to 2.3 fold after 6 h, to reach basal levels upon 24 h of exposure (Fig. 1, panel A). Treatment of Caco-2 cells with the LM3-CC1 strain failed to induce TLR-2 expression as monitored up to 24 h exposure (Fig. 1, panel A).

Expression of TLR-4 was induced of 1.6 fold after 3 h exposure to LM3 wild type, and decreased to basal levels upon 24 h exposure (Fig. 1, panel B); *L. plantarum* LM3-CC1 stimulated a similar level of TLR-4 expression in Caco-2 cells (Fig. 1, panel B).

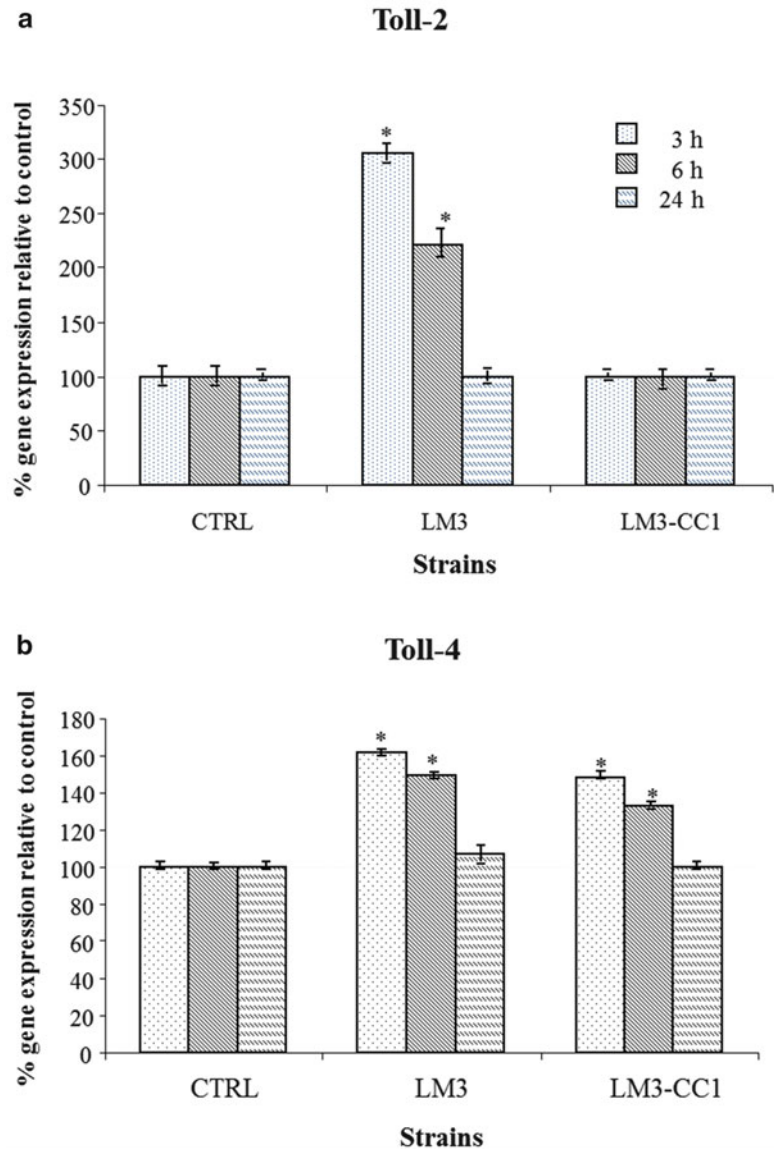
### 3.2 *L. plantarum* LM3-Induced HBD-2 Expression in Caco-2 Cells

To determine whether human colon epithelial cell lines constitutively express  $\beta$ -defensins, RNAs from un-stimulated Caco-2 cells were analysed by Real Time PCR using HBD-1, HBD-2 and HBD-3 specific primers. Caco-2 cells constitutively expressed a low level of mRNA for HBD-1 and HBD-3, whereas there was little if any constitutive expression of HBD-2 mRNA (data not shown).

When Caco-2 cells were exposed to *L. plantarum* LM3, HBD-2 expression was induced more than 3 times after 6 h of exposure, to reach again the basal level of expression upon 24 h treatment (Fig. 2). Exposure of Caco-2 cells to *L. plantarum* LM3-CC1 mutant strain produced a minor effect on HBD-2 expression,



**Fig. 1** Expression levels of *TLRs* genes in Caco-2 cells in the presence of *L. plantarum* LM3 and LM3-CC1. Comparison of the expression of TLR2 (a) and TLR4 (b) in Caco-2 cells after 3 h, 6 h, and 24 h exposure to *L. plantarum* LM3 or LM3-CC1. Expression levels are reported exposure. Data are expressed as percentage of the relative mRNAs in each sample compared to un-stimulated Caco-2 cells (CTRL), arbitrarily assigned as 100 %. The data are the mean values of three independent experiments (\* $p < 0.05$  relative to control sample)

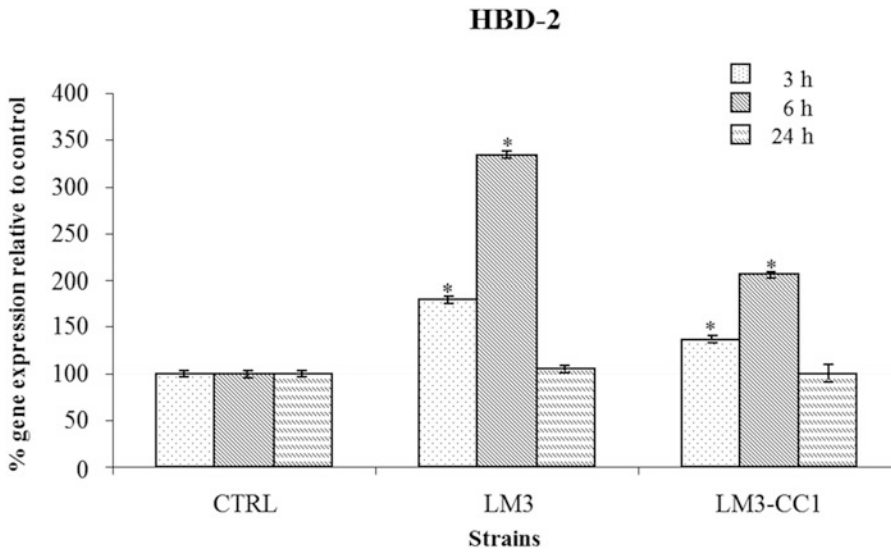


namely induction of only 2 times after 6 h treatment, to reach basal levels within 24 h exposure (Fig. 2).

### 3.3 *L. plantarum* LM3-Dependent Expression of Cytokines in Caco-2 Cells

We extended the analysis of the immunomodulatory effect determined by *L. plantarum* LM3 and

LM3-CC1 to the expression of cytokines in Caco-2 cells. Expression of the pro-inflammatory IL-6 was transiently induced more than five times after 6 h exposure to LM3 cells, to decrease to almost basal levels after 24 h treatment (Fig. 3, panel A). Exposure of Caco-2 cells to LM3-CC1 determined a much lower level of induction, being the expression of IL-6, after 6 h exposure, only twice as much the level detected in un-stimulated Caco-2 cells (Fig. 3, panel A). Moreover, expression of anti-



**Fig. 2** Expression levels of *HBD-2* genes in Caco-2 cells in the presence of *L. plantarum* LM3 and LM3-CC1. Comparison of the expression of HBD-2 in Caco-2 cells after 3 h, 6 h, and 24 h exposure to *L. plantarum* LM3 or LM3-CC1. Expression levels are

reported exposure. Data are expressed as percentage of the relative mRNAs in each sample compared to un-stimulated Caco-2 cells (CTRL), arbitrarily assigned as 100 %. The data are the mean values of three independent experiments (\*p < 0.05 relative to control sample)

inflammatory cytokines, namely IL-10 and TGFβ, were induced in Caco-2 cells exposed to *L. plantarum* LM3, reaching an expression of about 3,5 and 4 fold, respectively, after 24 h of treatment (Fig. 3, panels B and C). Exposure to LM3-CC1 produced an increase of IL-10 and TGFβ of 1.8 and 2.4 fold, respectively, after 3 h of treatment, and the level of induction remained mostly stable up to 24 h of treatment (Fig. 3, panels B and C).

observed at alkaline pH, which is a condition encountered in the colon, while about 40 % reduction was observed at pH 4.0 (data not shown). A comparative analysis between the two strains under study was then performed for the ability to develop biofilm at pH 7.0. A decrease of more than three fold was found in the amount of biofilm developed by *L. plantarum* LM3-CC1 respect to the wild type (Fig. 4), suggesting the involvement of the EnoA1 adhesin in this process.

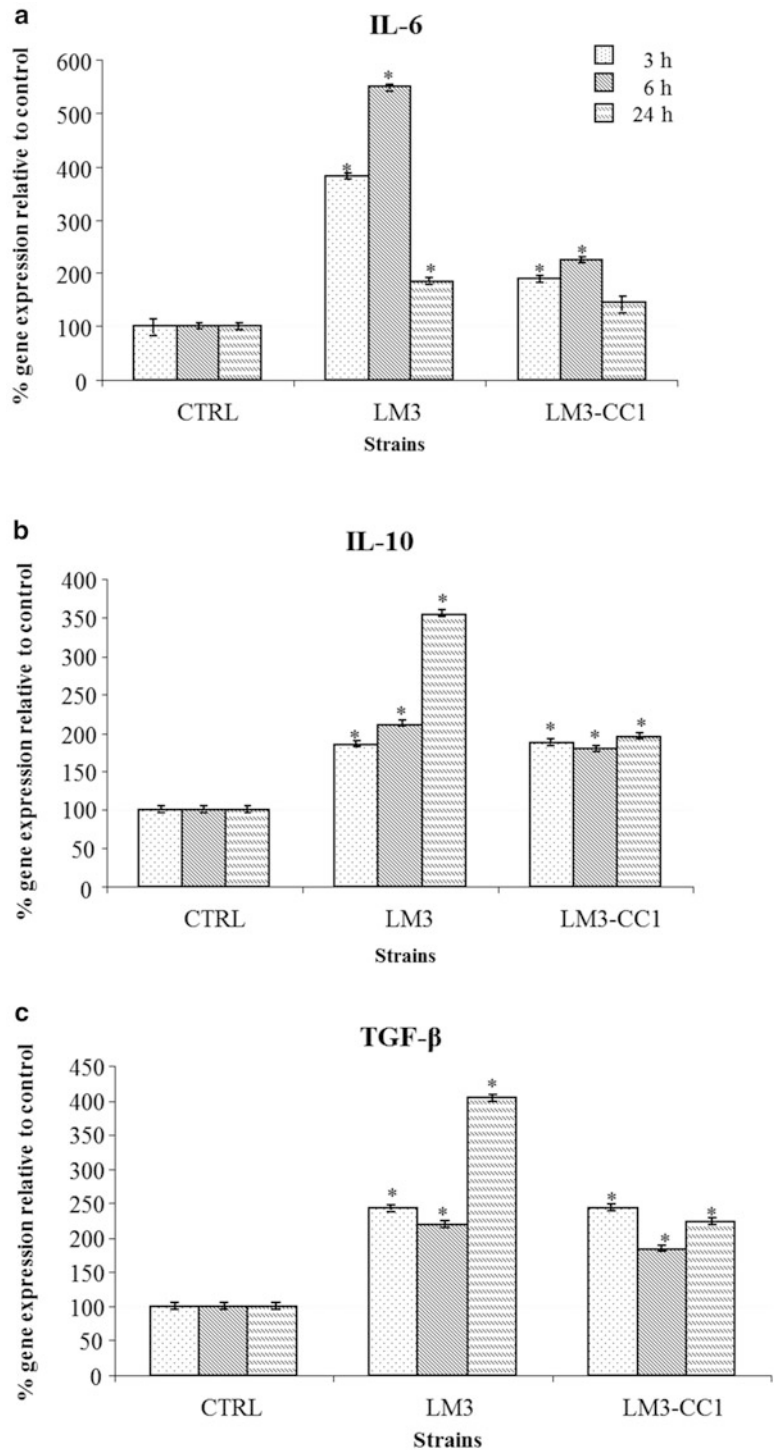
### 3.4 Surface Expressed EnoA1 Affects Biofilm Development in *L. plantarum*

The ability of a microorganism to develop biofilm in the gut environment plays a role for its persistence in the colon, being therefore considered a prerequisite for good performing probiotic strains (Ahrne and Hagslatt 2011). Development of *L. plantarum* LM3 biofilm was tested at different pH values, ranging from 4.0 to 8.0. Respect to the positive control, namely biofilm developed at pH 7.0, no significant variation was

## 4 Discussion

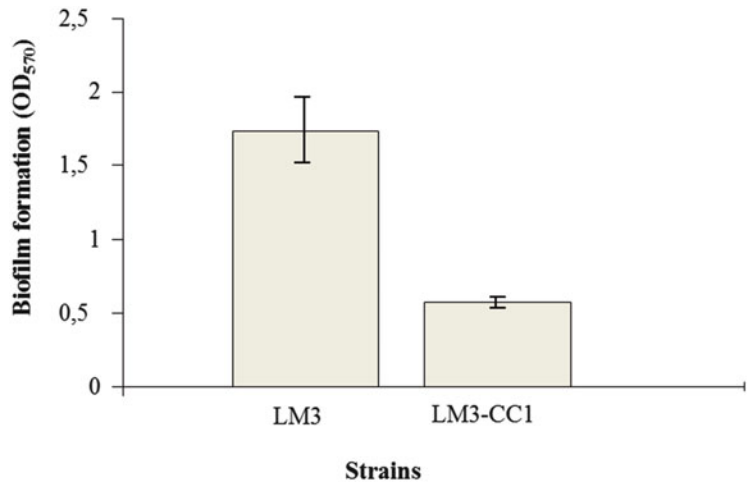
The gut microbiota is undoubtedly important in supporting a functional yet balanced immune system; processes leading to this balance can be emulated by transiently colonizing the gastrointestinal tract with appropriate strains of microbes that are delivered orally as probiotics and that influence the host microbiota and immune functions of the immune cells associated with the gut (Abreu et al. 2003).

**Fig. 3** Expression levels of pro-inflammatory and anti-inflammatory cytokines genes in Caco-2 cells in the presence of *L. plantarum* LM3 and LM3-CC1. Comparison of the expression of IL-6 (a), IL-10 (b), and TGF- $\beta$  (c) after 3 h, 6 h, and 24 h exposure to *L. plantarum* LM3 or LM3-CC1. Expression levels are reported exposure. Data are expressed as percentage of the relative mRNAs in each sample compared to un-stimulated Caco-2 cells (CTRL), arbitrarily assigned as 100%. The data are the mean values of three independent experiments (\* $p < 0.05$  relative to control sample)





**Fig. 4 Quantitative analysis of biofilm formation performed on *L. plantarum* LM3 and LM3-CC1.** Biofilm assay was performed on cultures of each strain grown to stationary phase (24 h) in MRS medium containing 0,2 % glucose. Results are the average of three independent experiments. The error bars represent standard deviation of the mean



Indeed, IEC is continually confronted by a variety of commensal bacteria and pathogen-associated molecular patterns (PAMPs). However, in the absence of pathogens, the lamina propria maintains a controlled state of inflammation through the development of a careful system of controlled TLR expression (Melmed et al. 2003). The expression of TLRs has been reported to increase during the course of infections or inflammatory bowel diseases (Cario and Podolsky 2000; Faure et al. 2001). Although TLR2 is normally present in epithelial cells, it plays a limited role in inflammation. It may be activated under conditions in which bacterial cell wall concentrations within the intestine are pathologically high (Naik et al. 2001; Otte et al. 2004). TLR4 is instead expressed at lower basal level respect to TLR2 in epithelial cells, and plays a role in the intestinal mucosal host defense against Gram-negative bacteria, being essential for LPS detection (Vinderola et al. 2005). Probiotic strains have been shown to counteract the inflammation process stimulated by pathogens with induction of anti-inflammatory cytokines secretion by a TLR2 dependent pathway, and induction of pro-inflammatory and anti-inflammatory cytokines secretion by a TLR4 dependent pathway; however, probiotics were shown to stimulate higher levels of TLR2 respect to TLR4 in epithelial cells (Bermudez-Brito et al. 2012;

Villena and Kitazava 2014). Moreover, some probiotic lactobacilli induce HBD-2 expression in epithelial cells, but they are not affected by the antimicrobial effect of HBD-2, which appears to be specific for pathogens (Schlee et al. 2007).

In this work we studied the immunomodulatory properties of *L. plantarum* LM3, by analysing expression of TLR2 and TLR4 mediators, IL-6 pro-inflammatory and IL-10 and TGF-beta anti-inflammatory cytokines, and the HBD-2 defensin in Caco-2 cells stimulated with *L. plantarum*.

Our results suggest that *L. plantarum* LM3 may use TLR-2, and to a lower extent TLR-4, to send immune signals to IEC, and the immune signals released would be closer to those of inflammation. This represents a mechanism that may impact on host immunity and disease development (Vinderola et al. 2005). Moreover, we showed that *L. plantarum* LM3 is able to induce HBD-2 expression. The time-dependence experiments showed a similar pattern as already described in previous studies with a maximum of HBD-2 induction after 6 h of incubation (Wehkamp et al. 2004). The interest in anti-bacterial peptides, as an innate mechanism of defense against bacteria, has increased in recent years. Antimicrobial peptides (AMPs) act mainly by damaging or destabilizing cytoplasmic membrane or at cytoplasmic level by inhibiting essential cellular processes. Indeed, recent evidences

suggest that probiotic bacteria may stabilize gut barrier function against pathogenic luminal bacteria through the induction of antimicrobial peptides, such as human  $\beta$ -defensins (HBDs) (Schlee et al. 2007; Schlee et al. 2008; Hugo et al. 2010). In particular, HBD-2 has been shown to be induced in the gut, and up-regulated in inflammatory bowel disease (Wehkamp et al. 2004; Schlee et al. 2008).

We also showed that *L. plantarum* LM3 induces transient expression of IL-6 in Caco-2 cells. The transient production of pro-inflammatory cytokines has been already reported for probiotic strains (Ukena et al. 2005; Ruiz et al. 2005). Indeed, probiotic bacteria induce the production of IL-6, necessary for T cell-independent clonal expansion of IgA producing-B cells, thus protecting epithelia from attachment of pathogens (Boirivant and Strober 2007). Moreover, our results indicate that IL-10 and TGF-beta anti-inflammatory cytokines were induced, reaching about four-fold increase after 24 h exposure. IL-10 has pleiotropic effects in immunomodulation and inflammation, being an essential immune-regulator in the intestinal tract; it is able to inhibit the synthesis of pro-inflammatory cytokines as well as to suppress the antigen presentation capacity of antigen presenting cells (Grimbaldeston et al. 2007). TGF-beta is involved in the control of tolerance through modulation of immune-cells proliferation, thus regulating initiation and resolution of inflammatory response (Li et al. 2006). Indeed, transient expression of pro-inflammatory and expression of anti-inflammatory cytokines in epithelial cells challenged with probiotics have been related to beneficial effects of these bacterial strains, representing a mechanism for control of pathogen invasions and a way for resolution of inflammatory response (Li et al. 2006; Villena and Kitazava 2014).

The ability of a microorganism to interact with host epithelial cells has been related to expression of proteins on cell surface. Among these, a large number of enzymes of the central metabolism in bacteria were shown to have additional functions in dependence of their localization; in particular some glycolytic enzymes were

shown to be expressed on cell surface and to be involved in binding to host cells and proteins (Henderson and Martin 2013; Kainulainen and Korhonen 2014). In previous works we have shown that the EnoA1 alfa-enolase of *L. plantarum* LM3 is surface expressed and mediates interaction with fibronectin, plasminogen and Caco-2 cells (Veiga-Malta et al. 2004; Adrian et al. 2015). By means of comparative analysis between *L. plantarum* LM3 and its isogenic mutant strain *L. plantarum* LM3-CC1, carrying a deletion in the *enoA1* gene, here we show that EnoA1 is involved in stimulation of TLR2 expression; indeed, Caco-2 cells challenged with LM3 wild type showed a three fold increase in TLR2 expression, while cells exposed to the LM3-CC1 mutant strain showed the same basal level found in the control sample. On the contrary, the absence of EnoA1 did not affect stimulation of TLR4 in Caco-2 cells challenged with LM3-CC1. Stimulation of cytokines and HBD2 is only partially affected by the absence of EnoA1, probably due to their already described double dependence on TLR2 and TLR4 pathways (Vora et al. 2004; Bermudez-Brito et al. 2012). We can not exclude that additional TLR pathways may be involved in *L. plantarum* immunomodulation.

We also found that the absence of EnoA1 lowered the ability to develop biofilm in *L. plantarum* LM3-CC1. Adhesins have a critical role in development of biofilms, important for persistence of probiotic strains in the colon (Ahrne and Hagslatt 2011). Moreover, it was shown that *Lactobacillus reuteri* biofilms have a higher protective effect on human THP-1 cells respect to the planktonic counterpart (Jones and Bersalovic 2009). Effects of treatments based on probiotics administration to counteract biofilm-associated infections have been recently reviewed (Vuotto et al. 2014).

In summary, here we show evidence for the role of surface expressed enolase in immunostimulation of Caco-2 cells exposed to *L. plantarum* LM3. Enolase has been described as an important antigen involved in virulence of many pathogens (Henderson and Martin 2013; Pancholi 2001; Dinis et al. 2009). Knowledge about enolase-

mediated activation of pathways involved in protective immunomodulation triggered by probiotics may give an insight on their competitive exclusion mechanisms against pathogens.

**Competing Interests** The authors declare that they have no competing interests.

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